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9/pnts.

09/674496

WO 99/58695

PCT/FR99/01085

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422 Rec'd PCT/PTO 13 NOV 2000

USE OF A POLYPEPTIDE DERIVED FROM A PA1b LEGUME
ALBUMEN AS INSECTICIDE

The present invention relates to insecticidal
5 proteins and to the use thereof for protecting plants,
and in particular cereals, their seeds and products
derived from them, against insect pests.

Insects which are pests for cereal seeds are to
be found in various families, in particular among
10 Coleoptera, Lepidoptera and Homoptera. Among
Coleoptera, mention will be made in particular of grain
weevils (*Sitophilus oryzae*, *Sitophilus zeamais*,
Sitophilus granarius), and of *Tenebrio* spp.,
Rhyzopertha dominica, *Trogoderma* spp. and *Tribolium*
15 *confusum*. Among Lepidoptera, mention will be made in
particular of *Sitotroga cerealella* and *Ephestia*
kuehniella.

Pests for cereal seeds are among the main
enemies of the crops which they attack in the field (at
20 least in hot regions), and especially in storage silos;
they may also attack transformed products which are
derived from cereals (for example, flours, semolinas,
etc). These insects cause very significant damage and,
each year, cause the destruction of a large portion
25 (which can come close to 25%) of the world harvest of
cereals harvested each year.

In order to combat these insects, various
methods have been recommended. The use of insecticides
(LINDANE®, then MALATHION® and ethylene bromide) is
30 currently being challenged because of the problems
posed by the presence of residues of these products in
food. In addition, resistance to these products has
appeared in many target insects, which makes their use
less and less effective. In order to replace these
35 insecticides or limit their use, various methods have
been proposed [for review, cf for example F.H. ARTHUR,
J. Stored Prod. Res., 32, pp. 293-302, (1996)]. The
methods which are currently the most developed are

physical methods, such as the cooling of silos or storage under CO₂ or under nitrogen; these methods are, however, expensive and their use, which requires great technological sophistication, is delicate; they are
5 therefore not applicable everywhere.

Another type of approach, which is the subject of much research, consists in producing transgenic plants expressing one or more gene(s) which confer(s) on them resistance against insect attack. However, this
10 approach requires the availability of suitable genes, which must also be acceptable both for the environment and by consumers.

Most insects exhibit more or less strict food specificity; it is in this way that cereal seeds are
15 attacked by grain weevils (*Sitophilus oryzae*, *Sitophilus zeamais*, *Sitophilus granarius*) which do not attack legume seeds; conversely, other pests, such as bruchid beetles, attack legumes but not cereals.

Previous studies by the inventors' team
20 [DELOBEL and GRENIER, J. Stored Prod. Res., 29, pp. 7-14, (1993)] have shown that the three species of *Sitophilus* mentioned above can develop on chestnuts or acorns, but that, conversely, they die rapidly on split peas, this mortality being consecutive to the
25 consumption of the peas by these weevils.

The inventors have undertaken to investigate the toxic substance responsible for this mortality. It is, moreover, known that legumes contain several entomotoxic substances and that, in diverse species of
30 insects for which legumes are toxic, there exist natural subpopulations which are more or less resistant to the toxicity of the legumes.

For example, in the case of grain weevils, a test carried out by the inventors' team on 90 strains
35 of different geographical origins has shown that 4 strains belonging to the *Sitophilus oryzae* species include individuals capable of surviving to the adult stage on split peas; conversely, no strain having this

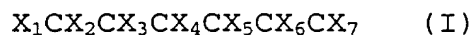
ability has been revealed in the *Sitophilus zeamais*, or *Sitophilus granarius* species; the study of the genetic determinism of this resistance has shown that this property is monogenic, recessive and autosomal [GRENIER
5 et al., Heredity, 79, pp. 15-23, (1997)].

The inventors have selected a strain of *S. oryzae* which is homozygous for this resistance gene, and have used this strain to investigate the toxic substance with respect to which the mechanism of
10 resistance encoded by this gene is expressed.

The inventors have thus noted that this toxicity is associated with isoforms of a protein which has a sequence similar to that of the PA1b pea albumin described by HIGGINS et al. [J. Biol. Chem., 261(24),
15 pp. 11124-11130, (1986)], and which shows strong similarity (65% identity) with soybean leginsulin [WATANABE et al., Eur. J. Biochem., 15, pp. 224:1-167-72, (1994)]. No entomotoxic property had until now been associated with the PA1b protein, with leginsulin or
20 with other homologous proteins.

The alignment of the sequence of one of the isoforms of the protein purified by the inventors, with those of the pea PA1b protein, published by HIGGINS et al., and of soybean leginsulin, published by WATANABE
25 et al., is represented in Figure 7. These 3 sequences include in particular 6 cysteine residues which occupy conserved positions.

A subject of the present invention is the use, as an insecticide, of a polypeptide comprising a
30 sequence which satisfies the following general formula (I):



in which C represents a cysteine residue, X_1 represents an amino acid or a sequence of 2 to 10 amino
35 acids, X_2 represents an amino acid or a sequence of 2 to 5 amino acids, X_3 represents a sequence of 4 to 10 amino acids, X_4 represents a sequence of 3 to 10 amino acids, X_5 represents an amino acid or a sequence of 2 to 4

amino acids, X_6 represents a sequence of 7 to 15 amino acids, and X_7 represents an amino acid or a sequence of 2 to 10 amino acids.

Preferably, X_1 represents a dipeptide, X_2 represents a tripeptide, X_3 represents a heptapeptide, X_4 represents a tetrapeptide, X_5 represents an amino acid, X_6 represents a nonapeptide, and X_7 represents a pentapeptide.

Advantageously:

- 10 - X_1 satisfies the sequence y_1y_2 in which y_1 and y_2 each represent an amino acid chosen from alanine, serine, glycine and threonine, or y_1 represents an amino acid chosen from alanine, serine, glycine and threonine, and y_2 represents glutamic acid or aspartic acid; and/or
- 15 - X_2 satisfies the sequence $y_3y_4y_5$ in which y_3 represents glutamine or asparagine, and y_4 and y_5 each represent an amino acid chosen from alanine, serine, glycine, threonine, valine, leucine, isoleucine and methionine; and/or
- 20 - X_3 satisfies the sequence $y_6y_7y_8y_9y_{10}y_{11}y_{12}$ in which y_6 represents an amino acid chosen from alanine, serine, glycine and threonine, y_7 , y_{11} and y_{12} each represent proline, y_8 represents an amino acid chosen from phenylalanine, tryptophan and tyrosine, y_9 represents
- 25 aspartic acid or glutamic acid, and y_{10} represents an amino acid chosen from valine, leucine, isoleucine and methionine; and/or
- X_4 satisfies the sequence $y_{13}y_{14}y_{15}y_{16}$, in which y_{13} , y_{14} , y_{15} and y_{16} each represent an amino acid chosen from
- 30 alanine, serine, glycine and threonine, or y_{14} represents an amino acid chosen from alanine, serine, glycine and threonine, y_{13} and y_{15} each represent a basic amino acid, and y_{16} represents aspartic acid or glutamic acid; and/or
- 35 - X_5 represents a basic amino acid; and/or
- X_6 satisfies the sequence $y_{17}y_{18}y_{19}y_{20}y_{21}y_{22}y_{23}y_{24}y_{25}$, in which y_{17} , y_{19} , y_{21} and y_{23} each represent an amino acid chosen from valine, leucine, isoleucine and methionine,

y₁₈ represents proline, y₂₀ and y₂₄ each represent an amino acid chosen from alanine, serine, glycine and threonine, y₂₂ represents an amino acid chosen from valine, leucine, isoleucine, methionine, phenylalanine, tryptophan and tyrosine, and y₂₅ represents an amino acid chosen from phenylalanine, tryptophan and tyrosine; and/or

- X₇ satisfies the sequence y₂₆y₂₇y₂₈y₂₉y₃₀ in which y₂₆ represents a basic amino acid or an amino acid chosen from valine, leucine, isoleucine and methionine, y₂₇ represents asparagine or glutamine or a basic amino acid, y₂₈ represents proline, and y₂₉ and y₃₀ each represent an amino acid chosen from alanine, serine, glycine and threonine.

According to one preferred embodiment of the present invention, the polypeptide used as an insecticide shows at least 40%, preferably at least 60%, homology with any one of the isoforms of a PA1b albumin.

For the purpose of the present invention, the term "PA1b albumin" is intended to mean not only any isoform of the pea PA1b protein, but also any protein of the same family which is present in other plants and which can especially be purified from seeds of legumes, in particular legumes of the Cesalpinaceae, Mimosaceae or Fabaceae family, or of the Meliaceae family, such as *Khaya senegalensis*.

Polypeptides which can be used in accordance with the invention may be natural polypeptides, for example leginsulins of legumes, such as the soybean leginsulin described by WATANABE et al.; they may also be artificial polypeptides, the sequence of which is derived from that of a PA1b by adding, deleting or substituting a small number of amino acids. It is possible to use, for example, polypeptides comprising a sequence which satisfies the general formula (I), or a portion of this sequence which corresponds to the region involved in the insecticidal activity. This

active peptide can optionally be fused, at its N-terminal end and/or at its C-terminal end, with another peptide sequence.

5 These polypeptides can be obtained by conventional methods, known per se, for example by peptide synthesis, or by genetic engineering, by expressing, in a suitable host cell, a sequence encoding the desired polypeptide. They can also, in the case of natural polypeptides, such as PA1b and
10 leginsulin, be purified from seeds of plants such as legumes or Meliaceae.

In accordance with the invention, the polypeptides comprising a sequence of general formula (I) can be used as the only active principle of an
15 insecticide, or combined with one or more other active principles. They can be used in particular for combating insects which are pests for cereal seeds, and also for combating plant-feeding insects, such as the lepidoptera *Mamestra brassicae* or *Ostrinia nubilalis* or
20 the coleoptera *Chrysomelidae*, for instance *Phaedon cochleariae* or *Curculionidae*, for instance *Anthonomus grandis*, or combating phloem-feeding insects such as aphids.

Furthermore, the inventors have noted that the
25 PA1b protein conserves its insecticidal activity for several years in dry seeds, and that this activity is not affected by heating to 100°C.

In addition, this protein is not toxic for humans or higher animals; it is present in the legumes
30 which form part of their conventional diet.

The polypeptides of general sequence (I) are particularly suitable for protecting, especially during storage, seeds, flours or transformed products which are derived therefrom.

35 For the implementation of the present invention, the concentration of the polypeptide of sequence (I) in the product to be protected (plant, seeds or derived products) is generally from 10 µmol/kg

to 100 mmol/kg (or from 10 μ M to 100 mM), and advantageously from 50 μ mol/kg to 10 mmol/kg (or from 50 μ M to 10mM).

According to one preferred embodiment of the present invention, the product to be protected is treated with a preparation comprising said polypeptide. This polypeptide can, for example, be in the form of a purified preparation or of an enriched fraction, which can in particular be obtained from seeds of plants which product said polypeptide naturally, or from cultures of cells which express a gene encoding this polypeptide.

According to another preferred embodiment of the present invention, a transgenic plant is produced which is transformed with at least one gene encoding said polypeptide, and which expresses the latter in at least one of its tissues or organs.

The present invention also encompasses the transgenic plants produced in this way; advantageously, said plants are cereals.

These plants can be obtained by the conventional techniques of plant transgenesis, which are known per se.

It is thus possible to obtain, in a plant, ubiquitous expression and/or expression and/or overexpression in certain tissues or organs (for example in seeds) of a polypeptide of sequence (I), and as a result, to protect the plant, tissue or organ concerned against attacks by insects for which this polypeptide is toxic. In particular, the expression of a polypeptide of sequence (I) in the seeds makes it possible to protect them, even after harvest, as well as the transformed products and flours obtained from these seeds.

The present invention will be more clearly understood with the aid of the following description which refers to nonlimiting examples, describing the

purification, and illustrating the insecticidal properties, of a legume PA1b albumin.

EXAMPLE 1 : DEMONSTRATION OF THE TOXICITY OF VARIOUS LEGUME MEALS FOR CEREAL WEEVILS

5 The toxicity of meals from various legumes was tested on weevils (*Sitophilus oryzae*). The experiments were carried out in parallel on wild-type animals (sensitive strain S), and on mutants surviving feeding on peas (resistant strain R).

10 The weevils (*Sitophilus oryzae*) are bred in a chamber regulated at 27.5°C and 70% relative humidity. One-week-old adults are removed from these mass breeding colonies for the tests. For each test, experimentation is carried out on batches of 30
15 insects, and daily mortality is noted.

 Balls of meal are kneaded with water, left to dry for 24 h and used for feeding the weevils. The gray wheat flour used is supplemented with various proportions of legume meal, sieved using a mesh size of
20 0.2 mm.

 The dose-response curves for weevil mortality were obtained using various doses of each meal to be tested. The results are processed using the "Toxicologie" [Toxicology] program [FEBVAY and RAHBÉ,
25 "Toxicologie", un programme pour l'analyse des courbes de mortalité par la méthode des probits sur MacIntosh ["Toxicology", a program for analyzing mortality curves using the probits method on a MacIntosh computer], Cahiers Techn. INRA, 27, pp. 77-78 (1991)]. This
30 program uses the transformation of the cumulative mortalities into probits, and determines the regression curve equation and the concentration for 50% lethality. These values are determined after exposure for 4 and 7 days.

35 In addition, for each concentration of pea meal, the times for 50% lethality (LT50) for the sensitive strain S are also calculated. The calibration curve thus established makes it possible to determine,

in the remainder of the experiments, for each meal or meal fraction tested, the equivalent concentration of pea meal (as % of pea in the wheat). This curve is given in Figure 1.

5

Pea meal toxicity:

Figure 2 shows the cumulative mortality for adults of the sensitive strain S of *Sitophilus oryzae*, on pea (◊), and on wheat (□), as a function of the feeding time in days. These results show that the cereal weevils are rapidly killed on pea: in 8 days, between 90 and 100% of the adults are dead.

Figure 3 shows the mortality at 6 days of *Sitophilus oryzae*, for balls containing various concentrations of pea meal; the resistant strain (R) and the sensitive strain (S) are compared. The dose/response curve thus established shows that, for the sensitive strain (S), from 10% of pea meal upward, 70% mortality is observed in 6 days (and 100% in 14 days). In the same period of time, the resistant strain (R) is not affected.

Toxicity of other legume meals:

Among the legume seeds used in the human diet, 10 were tested for their action on the sensitive and resistant weevils.

Balls containing 80% of legume meal and 20% of wheat flour were used. Figure 4 illustrates the cumulative mortality of the *Sitophilus oryzae* weevils, resistant strain R (◻) or sensitive strain S (◻), measured after 5 (4 A), 7 (4 B), 14 (4 C) and 20 (4 D) days of feeding on cowpea (*Vigna unguiculata*) white (1) and red (2) variety bambora groundnut (3 : *Vigna subterranea*), lentil (4 : *Lens esculenta*), French bean (5 : *Phaseolus vulgaris*), mung bean (6 : *Vigna radiata*), adzuki bean (7 : *Vigna angularis*), broad bean (8 : *Vicia faba*), chickpea (9 : *Cicer arietinum*), and lupin (10 : *Lupinus albus*).

The results show that, at 7 days, all the legumes are toxic for the sensitive strain, even though *Vigna subterranea* and *Cicer arietinum* have not yet killed all the insects which live thereon; conversely, the resistant strain shows no or very little mortality. It can therefore be concluded that the same mechanism causing the toxicity is present in all these legumes; this mechanism appears in particular to be predominant in *Vigna subterranea*, *Vigna radiata* and *Cicer arietinum*.

However, examination of the mortalities at 14 and 20 days on certain legumes reveals, for the resistant strain, higher or lower mortality which must, therefore, be attributed to other mechanisms; this is in particular the case on *Phaseolus vulgaris* and on *Vigna angularis*.

EXAMPLE 2 - PURIFICATION AND IDENTIFICATION OF THE SUBSTANCE RESPONSIBLE FOR THE TOXICITY IN PEAS

Preparation of a protein fraction enriched in albumin (SRA1).

The fraction enriched in albumin is prepared on a pilot scale according to the protocol developed by CREVIEU et al. [Nahrung, 40(5), pp. 237-244, (1996)].

The pea meal (10 kg) is mixed, with stirring, with 140 liters of acetate buffer (pH 4.9), the mixture is centrifuged at 7500 rpm and the supernatant is subjected to ultrafiltration on an M5 membrane, at a temperature which does not exceed 25°C. The retentate is subject to diafiltration on the same membrane, the new retentate is centrifuged at 6000 rpm for 20 min and the supernatant is lyophilized. The powder obtained (SRA1), which represents on average 1% of the mass used at the start, is used for the subsequent purifications.

At each step of the purification, the toxicity of the various fractions is determined according to the protocol described in Example 1 above.

Anion exchange chromatography

10 g of SRA1 are suspended in 100 ml of a 60% methanol solution and stirred for 1 hour at 4°C. After centrifugation (30 min, 9000 g, 4°C), the supernatant is recovered and then the methanol present is removed in a rotary evaporator. The volume is then readjusted to 100 ml with water and a 1M Tris-HCl buffer (pH 8.8) so as to obtain a final Tris-HCl concentration of 50 mM. The soluble proteins are fractionated by anion exchange chromatography on a DEAE SEPHAROSE FAST FLOW column (120 × 50 mm). The proteins adsorbed are eluted with a 50% concentration of buffer B (50 mM Tris-HCl, pH 8.8; 500 mM NaCl) in buffer A (50 mM Tris-HCl, pH 8.8). The elution flow rate is 20 ml/min and the fractions collected have a volume of 80 ml. The proteins are detected by absorption at 280 nm.

The chromatogram is shown in Figure 5. The concentration of buffer B is indicated by the broken line. The 80 ml fractions corresponding to the peaks are pooled into two main fractions, DEAE NA and DEAE 1, indicated on the chromatogram by the horizontal lines. The nonadsorbed fraction (DEAE NA) contains all the toxicity.

This fraction is dialyzed against water for 72 hours and then lyophilized. Approximately 450 mg of the DEAE NA fraction are thus obtained.

Semipreparative reverse phase HPLC chromatography.

The DEAE NA fraction obtained after anion exchange chromatography is fractionated by reverse phase HPLC (RP-HPLC) chromatography on a HYPERSIL column (250 × 10.5 mm) filled with C18-aliphatic-chain-grafted 5 µm 300 Å NUCLEOSIL. For each chromatography, 15 mg of proteins are loaded on to the column. The elution flow rate is 3 ml/min and the proteins are detected by absorption at 220 nm. The proteins are eluted with a gradient of buffer B (0.04% of trifluoroacetic acid in acetonitrile) in mixture A (0.04% of trifluoroacetic acid in water) according to

the following sequence: t=0 min, 40% of B; t=5 min, 40% of B; t=17 min, 48% of B; t=18 min, 80% of B; and t=23 min, 80% of B.

The chromatogram is illustrated in Figure 6.
5 The acetonitrile gradient is represented by the broken line. The toxicity is located only in the peaks F1 and TP; the fractions corresponding to these peaks which have been collected are represented on the chromatogram by horizontal lines.

10 Thirty successive chromatographies, corresponding to an injected amount of DEAE NA of 450 mg, were carried out. The fractions were pooled and then lyophilized after evaporating off the acetonitrile and the trifluoroacetic acid in a SPEED VAC. 4 mg of
15 the TP fraction and 5 mg of F1 were thus obtained. These fractions were then analyzed by reverse phase HPLC (RP-HPLC) chromatography.

Reverse phase HPLC chromatography

The control of the purity of the proteins of
20 the F1 and TP fractions is carried out by reverse phase HPLC chromatography on an INTERCHROM column (250 x 4.6 mm) filled with C18-aliphatic-chain-grafted 5 µm 100 Å NUCLEOSIL. The elution flow rate is 1 ml/min and the proteins are detected by absorption at 220 nm.

25 The proteins are eluted in 45 minutes with a linear gradient of 0 to 50% of mixture B (0.04% of trifluoroacetic acid in acetonitrile) in mixture A (0.04% of trifluoroacetic acid in water).

This analysis shows that the TP fraction
30 contains only the toxic protein TP. The F1 fraction is more complex and contains two major polypeptides.

Characterization of the proteins present in the fractions TP and F1

35 The mass determinations were carried out by electrospray mass spectrometry (ES-MS). The mean masses calculated from 2 estimations are 3741.1 Da in the case of TP, and 3736 and 3941 Da for the polypeptides of the TF fraction.

The number of cysteines free and involved in disulfide bridges was determined by alkylating the protein with iodoacetamide, before and after reduction, and comparing the retention times, by RP-HPLC, and the
5 masses, by ES-MS, of the alkylated proteins with the native protein.

The alkylated nonreduced protein has both a retention time and a mass identical to that of the native protein. On the other hand, the protein which is
10 reduced and then alkylated has a retention time which is clearly different from that observed for the native protein (30 min instead of 42 min) and a mass of 4089.9 Da.

It appears therefore that this protein contains
15 6 cysteines, which are all involved in 3 disulfide bridges.

Complete sequence of the TP protein

The complete sequence of the TP protein was established. The mass calculated from the 37 residues
20 of the protein is 3741.4 Da, which is identical, give or take the measurement error, to that determined by mass spectrometry (3741.1 Da) for the native protein. The value calculated for the protein alkylated with iodoacetamide (4090 Da) is also equivalent to that
25 obtained experimentally (4089.9 Da). These results demonstrate the absence of post-translational modifications (glycosylations, phosphorylations, etc.) of the protein.

The sequence of the TP protein shows very
30 strong homology with that of the PA1b pea albumin [HIGGINS et al, J. Biol. Chem, 261(24), pp. 11124-11130, (1986)]. The two sequences differ only by the replacement of the valine residue at position 29 in the TP protein with an isoleucine in PA1b. Strong
35 similarity (62% identity, 89% homology, determined with the aid of the MAC MOLLY program using the BLOSUM62 matrix) is also observed between the TP protein and soybean leginsulin [WATANABE et al., Eur. J. Biochem.,

15, pp. 224:1-167-72, (1994)]. In particular, the 6 cysteine residues, which play an essential role in the structure of the proteins, occupy conserved positions.

The comparison of these 3 sequences is shown in
5 Figure 7.

These results make it possible to conclude that the protein responsible for the resistance of pea to cereal weevils is similar to the PA1b protein described by HIGGINS. This protein is synthesized in the form of
10 a 130-residue preproprotein (PA1) which undergoes post-translational maturation releasing the PA1b protein and a 53-residue protein named PA1a [HIGGINS et al., J. Biol. Chem., 261(24), pp. 11124-11130, (1986)].

Sequencing of the first 10 N-terminal residues
15 of each of the toxic polypeptides of the F1 fraction was also carried out. The sequences obtained are identical to that of the N-terminal end of the TP protein. As, in addition, the masses of these polypeptides determined by ES-MS are very close to that
20 of TP, it appears that these polypeptides represent isoforms of TP.

EXAMPLE 3 - ACTIVITY AND STABILITY OF THE ENTOMOTOXIC PROTEINS EXTRACTED FROM PEAS

Activity:

25 The entomotoxic activity of the polypeptides of the TP fraction or of the F1 fraction was determined as described in Example 1 above; at the concentration of 1% in the wheat flour (3 mmol/kg), these polypeptides have a toxicity for the weevil which is equivalent to
30 that of pure pea meal. A concentration of 60 μ mol/kg is sufficient to prevent any infestation by the weevils.

Stability:

The polypeptides of the TP fraction or of the F1 fraction, extracted from dried seeds stored for
35 several years, conserve their entomotoxic activity. In addition, this activity is not affected by heating to 100°C.

Toxicity for various insects:

The toxicity of the TP protein for the flour moth *Ephestia kuehniella* (Lepidoptera) and for the aphid *Acyrtosiphon pisum* (Homoptera) was also tested.

5 The tests on the flour moth were carried out on first and second stage *Ephestia kuehniella* larvae fed on wheat flour balls containing various concentrations of the TP protein (In mmol per kg of wheat flour). The results are shown in Figure 8.

10 (O = survival at 0 days;
 ▲ = survival at 4 days;
 □ = survival at 10 days).

These results showed that this protein was very toxic, from the concentration of 0.25 mmol/kg upward.

15 The aphid *Acyrtosiphon pisum* (Homoptera) was fed on artificial medium containing various concentrations of the TP protein.

 (□ = 3.3 μ M;
 ▲ = 17 μ M;
20 ◆ = 46 μ M;
 ○ = 84 μ M;
 ■ = 100 μ M).

 The results, which are shown in Figure 9, show that considerable mortality appears from the
25 concentration of 46 μ molar upward, this mortality being total at 100 μ molar.